Dye residues in aquaculture products: Targeted and mass spectrometric approaches to track their abuse

1.1 Contexte

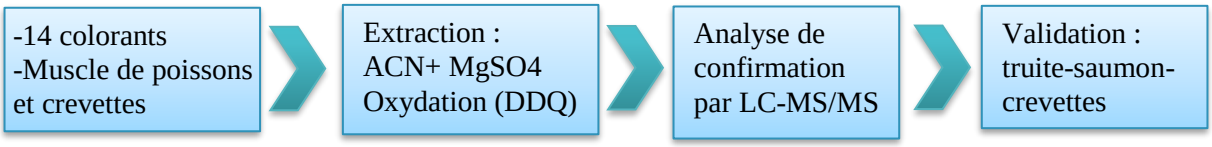
Ces résultats sont issus d'un projet intitulé Multicolor, mis en œuvre en partie à l'aide d'un travail réalisé par un étudiant de Master. Ce travail avait pour objectif d'initier une stratégie de surveillance élargie des colorants à usage thérapeutique par le développement d'approches analytiques ciblées et non ciblées. Le projet s'inscrivait dans un objectif méthodologique plus large que porte notre laboratoire en matière d'approches non ciblées pour la détection de marqueurs de traitement. Identifier un marqueur pertinent présente un intérêt dans plusieurs cas. Il permet par exemple d'identifier la signature biologique d'un traitement par un médicament aujourd'hui interdit dont le composé parental disparait rapidement après son administration à l'animal, ou encore de mettre en évidence cette signature imputable à un ensemble de molécules pharmacologiquement similaires. Pour cela, nous avons fait appel au savoir-faire de l'ONIRIS-Laberca qui a réalisé le traitement de données et les analyses statistiques multivariées. Ces données ont été reprises et complétées au laboratoire. Le projet Multicolor comportait ainsi 3 volets : le développement de l'approche analytique ciblée, une étude d'occurrence sur produits d'aquaculture du commerce en appliquant la méthode ciblée, et enfin l'approche de métabolomique non ciblée. Les deux approches, qui visaient soit à élargir le nombre de colorants ciblés dans le premier cas, soit à suivre des biomarqueurs appropriés dans le second cas, ont été étudiées de manière concomitante avec le même objectif d'améliorer la stratégie de surveillance des nouvelles pratiques illégales de traitements chimiques en aquaculture.

1.2 Méthodologie et principaux résultats

Méthodologie de l'approche ciblée :

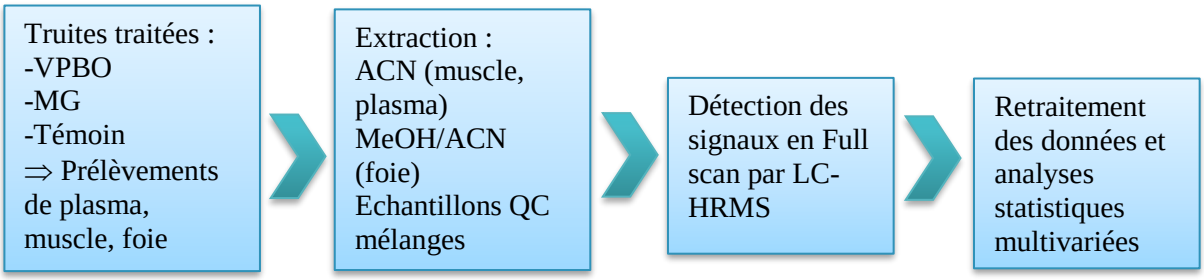
Une méthode LC-MS/MS comprenant une étape d'oxydation appliquée à la plupart des colorants sélectionnés a été développée et validée pour cibler l'analyse de 14 résidus chimiques appartenant à différentes familles de colorants. La méthode est appropriée pour la confirmation quantitative de 13 colorants dans la chair de poisson à des concentrations résiduelles de l'ordre de 1 µg/kg. Une étape d'oxydation a été intégrée à l'analyse afin de retransformer les substances

métabolisées et réduites en leuco-formes par les poissons, en leurs formes parentes, et de quantifier ensuite uniquement ces formes parentes. Tandis que chez certains TAMs les leuco-formes étaient déjà connues (la leucobase du vert de malachite par exemple), d'autres composés étaient probablement non connues, ou bien avaient fait l'objet d'une première identification récente et ne comportaient ainsi toujours pas le standard permettant de quantifier la forme réduite, comme c'est le cas pour le LBG. Les composés ciblés par la méthode comprenaient les principaux TAMs (MG, CV, BG, EV, PRRA), quelques autres dérivés des triarylméthanes (VBB, VBR, VPBO), des xanthènes (RHG), des phénothiazines (MB, AZB, NMB) et des phénoxazines (NBA). Ces colorants ont ensuite été recherchés sur des produits d'aquaculture collectés dans des commerces locaux.

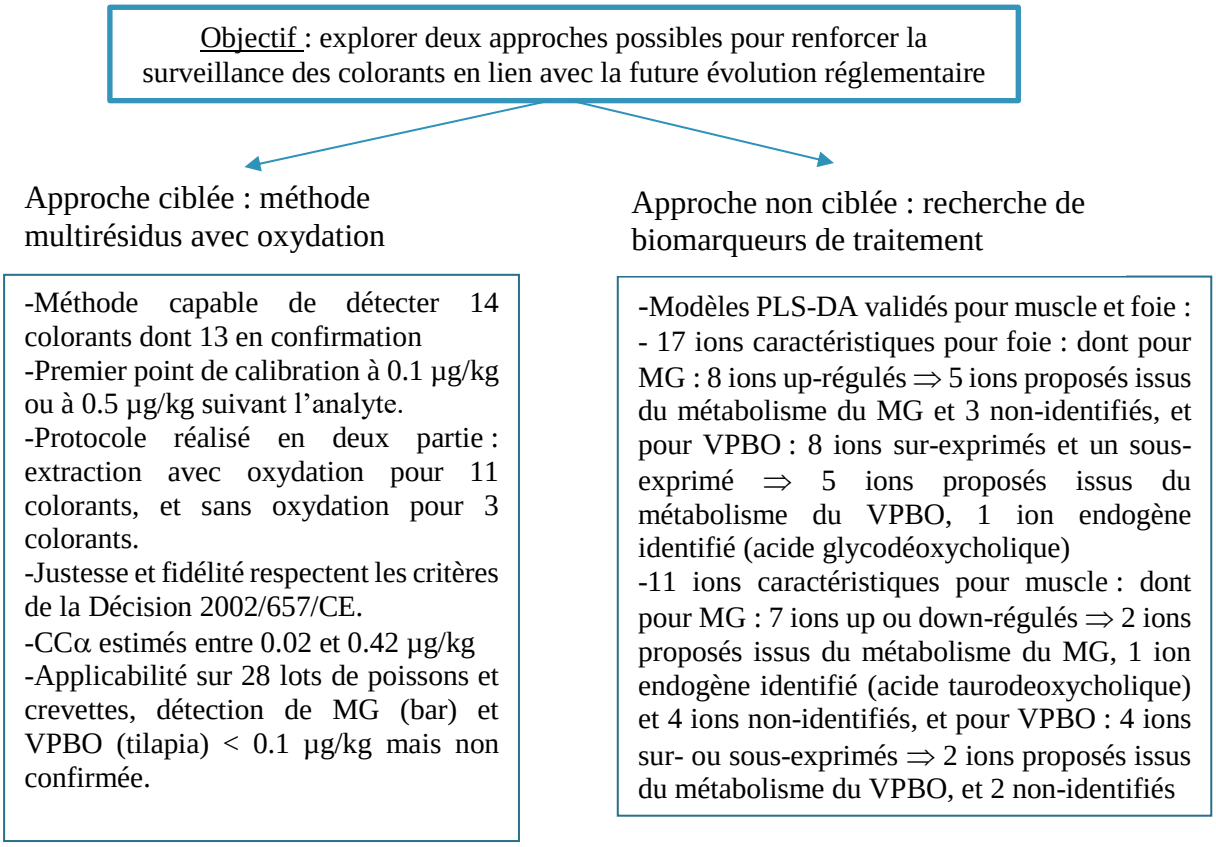


Méthodologie de l'approche ciblée :

Le deuxième objectif de ce projet était de rechercher tout marqueur de traitement des colorants au moyen d'une approche non ciblée basée sur la LC-HRMS en utilisant des échantillons biologiques de truites récoltés lors d'une expérimentation *in vivo*. Le principe de cette approche était de comparer les empreintes métaboliques obtenues avec des truites traitées à MG et celles obtenues avec des truites traitées à VPBO, avec pour objectif de détecter des marqueurs endogènes communs aux deux traitements ou encore de déterminer tout autre biomarqueur pertinent significatif d'un traitement par des colorants de type TAMs. Les informations extraites de l'analyse par LC-HRMS des ions caractéristiques provenant d'échantillons de muscles, de foie et de plasma ont été exploitées à l'aide de modèles statistiques multivariés. Elles ont révélé des modifications du métabolome de la truite générant certains métabolites directement liés aux 2 colorants mais également des substances endogènes perturbées suite au traitement.



Principaux résultats :



1.3 Conclusion

Les deux pistes explorées en vue d'étendre le contrôle des résidus de colorants interdits chez les produits d'aquaculture, pourraient se rejoindre et se compléter comme il est explicité en conclusion de l'article. La première piste consistait à déterminer si une méthode ciblée, incluant une oxydation pour pallier le manque d'informations et de standards analytiques pour tous les métabolites de tous les colorants concernés, pouvait être développée et validée avec une sensibilité suffisante pour tenir le challenge règlementaire autour du µg/kg. Les résultats ont montré que le protocole est applicable pour 11 colorants subissant l'étape d'oxydation et pour 3 colorants sans cette étape, car l'oxydation engendre probablement une dégradation de ces 3 composés. Les performances de la méthode sont satisfaisantes puisque les CCα sont inférieurs à 0.5 µg/kg. Les avantages de cette méthode sont le taux de recouvrement des colorants incluant les leuco-bases, connues et non connues, sa sensibilité, et sa facilité d'application. Les inconvénients sont d'une part, qu'elle inclut des colorants déjà suspectés mais pas de potentiels nouveaux colorants, et que d'autre part, l'étape d'oxydation correspond à une seule réaction de métabolisme, donc le champ des métabolites traceurs de traitement n'est pas entièrement

couvert. La deuxième piste consistait en l'investigation de métabolites endogènes marqueurs d'un traitement aux colorants, plus spécifiquement les TAMs et dérivés, en apportant des informations sur l'endo-métabolome des poissons.

Deux acides biliaires ont été subi une perturbation de leur voie d'expression chez la truite, mais aucun d'entre eux n'a pu être confirmé comme étant commun aux deux traitements issus des deux TAMs. Ceci laisse néanmoins présager d'un effet de ces colorants sur la voie de synthèse des acides biliaires. Cette étude a également mis en évidence des métabolites exogènes provenant directement d'une dégradation des colorants appliqués, dont les métabolites du VPBO, inconnus jusqu'ici. L'approche menant aux biomarqueurs d'effet d'un traitement (métabolites endogènes) mériterait d'être approfondie, et renforcée, afin de détecter d'autres biomarqueurs pouvant dans leur ensemble constituer un modèle prédictif de dépistage d'un traitement aux colorants. Enfin, ces deux pistes pourraient être combinées dans une même étude, afin d'obtenir une approche la plus exhaustive possible et générer des données d'occurrence par la méthode ciblée tout en étudiant l'impact de ces données d'occurrence sur des biomarqueurs comme les acides biliaires.

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Review

Dye residues in aquaculture products: Targeted and metabolomics mass spectrometric approaches to track their abuse

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ABSTRACT

The aim of the study was to initiate an exhaustive strategy of control by implementing both targeted and non-targeted metabolomics approaches. A LC–MS/MS method including an oxidative step for most of dyes was developed and validated to target the analysis of 14 residues belonging to different families of dyes. The method was suitable for the quantitative confirmation of 13 dyes at low ppb levels. The metabolomics approach objective was to compare fingerprints between farmed fish treated with malachite green and farmed fish treated with victoria pure blue bo. Analytical information on modifications in the metabolome of muscle, liver and plasma was exploited by HRMS following by multivariate statistics and revealed some direct or endogenous metabolites among relevant mass features contributing to the constructed models. These two approaches, either appropriate biomarkers either enlarged targeted dyes are explored concomitantly to help improving the strategy for tracking new illegal practices in aquaculture.

1. Introduction

Dyes are substances used in aquaculture production that have been determined by international regulatory agencies to pose health-related hazards to consumers. Besides the use of dyes in aquaculture as biocides, dyes like malachite green (MG), are intensely used in industry to color silk, wool, cotton, wiping paper and newspaper, and also as food coloring agent, food additive, medical disinfectant and anthelmintic (Fallah & Barani, 2014). The multiple applications, the availability, the low cost of dyes generate a persistent presence of them in environment (Belpaire, Reyns, Geeraerts, & Van Loco, 2015; Khodabakhshi & Amin, 2012). This occurrence worries the authorities because dyes are demonstrated to induce toxicological effects (Srivastava, Singh, Srivastava, & Sinha, 1995) in relation to electron transfer, oxidative stress or other physiological effects (Culp et al., 2002, 1999; Kovacic & Somanathan, 2014). Thus the use of malachite green as veterinary medicine in aquaculture is now for more than 15 years illegal in Europe (Commission Decision No 2004/25/EC). Recently, EFSA has confirmed a Reference Point for Action (RPA) of 2 $\mu g\,kg^{-1}$, expressed as the sum of chromic- and leuco-form for the analysis of MG (Contam- Malachite (Scientific opinion), 2016). MG is as of today the only one dye having been assigned a RPA in the EU. Despite their ban in many countries, malachite green and other triarylmethane dyes are still used in some fish farming practices around the world. Tao et al. (2011) reported positive samples of malachite green and of crystal violet in salmon production. Extraction from the RASFF (Rapid Alert Reports for Food and Feed in EU) for alerts due to malachite green and its metabolite residues in fish shows that they were regularly found in farmed trout between 2002 and 2013 (Fallah & Barani, 2014). Moreover, it was assumed that it would be possible that other dyes might be selected to replace the well-known triarylmethanes like malachite green or crystal violet (Reyns, Belpaire, Geeraerts, & Van Loco, 2014; Tarbin, Chan, Stubbings, & Sharman, 2008). Other compounds derived from the family of triarylmethanes as the Victoria blue sub-family, or other dyes including phenothiazines, and phenyl azoic derivatives are often suggested but with no evidence acknowledged yet. Regarding the Victoria pure blue BO, the RASFF was notified in 2010 a first evidence of contamination of farmed fish imported from Vietnam (2010.1372 notification). Currently only some triarylmethane dyes known for their potential usage in aquaculture have been included in the French official control method: i.e., the malachite green and the crystal violet with their respective leuco-metabolites, and the brilliant green. It is therefore

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necessary to start investigating the occurrence of residues of other families of dyes that were previously not forecast for their use in aquaculture. Some laboratories have already started to extend the scope of dyes in their analytical method regarding the only detection of malachite green, crystal violet and their leuco-metabolites. The multi-residue LC–MS/MS methods of Tarbin et al and of Reyns et al. (Reyns et al., 2014; Tarbin et al., 2008) include 13 and 14 dyes, respectively, allowing oxidation of the leuco-metabolites. In fact, malachite green and crystal violet when absorbed by fish are metabolized to form lipophilic reduced leucomalachite green (LMG) and leucocrystal violet (Thompson, Rushing, Gehring, & Lochmann, 1999). Hurtaud-Pessel, Couëdor, and Verdon (2011) have shown that brilliant green is also reduced in its leuco form. Some other dyes included in their method could also benefit from this oxidation that permits the quantitation regarding the only parent form. Another area to prospect on the occurrence of unknown used illegal drugs like dyes is to process metabolomics studies. For the characterization of complex biological samples, metabolomics is one of the promising strategies as it allows the production of a chemical pattern or fingerprint of the medicinal treatment. The specificity of the high resolution mass spectrometry (HRMS) facilitates the identification of the markers discovered through the elucidation of their chemical structures. The last decade provided the emergence of metabolomics strategies based on the observation of physiological perturbations in order to tackle drug abuse in animal breeding. Some metabolomics studies were implemented to study the effects of antibiotic treatment in animal tissues (Hermo, Gómez-Rodríguez, Barbosa, & Barrón, 2013; Hermo, Saurina, Barbosa, & Barrón, 2014) and to highlight some markers for a better food quality evaluation. Besides, drugs of abuse are more and more subject of studies in biological fluids to focus on changes of fingerprints (Arias et al., 2016; Dervilly-Pinel, Chereau, Cesbron, Monteau, & Le Bizec, 2015; Peng, Royer, Guitton, Le Bizec, & Dervilly-Pinel, 2017; Wu et al., 2015) and lead to metabolite identification or even descriptive and predictive models proposals.

In this study, we aim enlarging and improving the determination of dyes in aquaculture products by two complementary strategies. On the one hand, a multi-family method based on liquid chromatography and tandem mass spectrometry is developed to extend the scope of targeted dyes including an oxidizing step. Targeted compounds include the main triarylmethanes (malachite green, crystal violet, brilliant green, ethyl-violet, pararosaniline), and other triarylmethanes derivatives (victoria blue B, victoria blue R, victoria pure blue BO), xanthenes (rhodamine 6G), phenothiazines (methylene blue, azure B, new methylene blue), and phenoxazines (Nile blue A). On the other hand, the second objective is to determine any marker(s) of dye treatment through an untargeted approach based on the analysis by liquid chromatography coupled to high resolution mass spectrometry of the samples harvested after appropriate farmed fish experimentations. To the best of our knowledge, this is the first time that metabolomics investigation is set up for dyes contaminated treated animals. The objective was to compare the general metabolic fingerprinting obtained from fish treated with a reference triarylmethane, the malachite green, with the one of fish treated with a derivative of triarylmethane, the victoria pure blue BO, with a final aim of determining common or any other relevant biomarkers of dye treatment.

2. Experimental

2.1. Chemicals, reagents and solutions

The standard substances malachite green (MG, purity of 96.9%), leuco-malachite green (LMG, purity of 98.5%), crystal violet (CV, purity of 92.7%), leuco-crystal violet (LCV, purity unknown estimated at 100%), brilliant green (BG, purity of 86.0%), ethyl violet (EV, purity of 92.8%), azure B (AZB, purity of 90.6%), methylene blue (MB, purity of 91.0%), new methylene blue (NMB, purity of 89.8%), Nile blue A (NBA, purity of 92.0%), pararosalinine (PRRA, purity of 97.0%), victoria blue R (VBR, purity of 92.2%), victoria blue B (VBB, purity of 93.0%), victoria pure blue BO (VPBO, purity of 91.4%), rhodamine B (RHB, purity of 97.9%) and rhodamine 6G (RHG, purity of 95.8%) were purchased from Merck (Darmstadt, Germany). The internal standard (IS) crystal violet-d_6 (CV-d_6) was obtained from Witega (Berlin, Germany). Stock solutions of the individual substances with a concentration of 100 µg mL^{-1} were prepared in either methanol for MG, BG, VBB, VBR, PRRA, NBA, RHB, RHG or acetonitrile for CV, VPBO, MB, NMB, EV, AZB, CV-d_6 and were kept stable in the dark at −20 °C during at least 1 month. For the targeted method, the dyes were divided into 2 groups during the validation according to their sensitivity. Further working standard solutions of the substances and their mixtures with appropriate concentrations were also produced by dilution with acetonitrile and were prepared fresh each day. These working solutions were prepared without MG and CV to spike the matrix-fortified validation samples (SV). The internal standard solution CV-d_6 was prepared at 100 µg L^{-1} in acetonitrile. Deionised water was prepared with a Milli-Q purifier (Millipore, St.-Quentin-en-Yvelines, France). Ammonium acetate, formic acid and 2,3-dichloro-5,6-dicyanobenzoquinone were from Merck (Darmstadt, Germany); ascorbic acid and anhydric magnesium sulfate from VWR (Fontenay-sous-Bois, France). Acetonitrile and methanol obtained from Fisher Scientific (St.-Quentin-Fallavier, France) were high performance liquid chromatography (HPLC)-grade and solvents. The solution of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) was prepared at 5 mM L^{-1} in acetonitrile. The final solution of reconstitution was prepared by mixing 80% of acetonitrile and 20% of a solution of acid ascorbic at 1 g L^{-1}. The mobile phase A consisted of a mixture of ammonium acetate 10 mM L^{-1} and formic acid 0.1% in deionized water. The mobile phase B was 100% acetonitrile.

2.2. Targeted approach

2.2.1. Extraction procedure

Two grams of mixed muscle (fish or prawn) tissue were weighted into a clean centrifuge tube. The sample was spiked with an adequate volume from 100 to 400 µL of the working solution at 2 µg L^{-1} or 10 µg L^{-1} for less sensitive dyes. Then 50 µL of the internal standard solution and appropriate volumes of acetonitrile were added. The sample was vortex-mixed and let to stand for contact for 10 min in a dark place. Six mL of acetonitrile was added and then 0.5 g of $MgSO_4$ and the sample was again vortex-mixed to homogenize the material with the solvent. The sample was further placed on a mechanical rotary shaker for 10 min at 100 rpm and then centrifuged for 5 min at 4000 g refrigerated at 4 °C. A portion of 300 µL of the supernatant was transferred into a LC vial by filtration of the residue through a 0.45 µm syringe PVDF filter (Millex-HV, Merck Millipore, Guyancourt, France). This fraction was analysed by LC–MS/MS for the dyes NMB, PRRA and VPBO. 5 mL of the supernatant was subsequently transferred to a polypropylene tube and 2 mL of the DDQ solution was added. The sample was again placed on a mechanical rotary shaker for 30 min at 100 rpm. The sample was evaporated to dryness under gentle nitrogen flow at 50 °C. The residue was dissolved in the mixture solution for reconstitution (80% acetonitrile and 20% ascorbic acid), vortex-mixed and then centrifuged for 5 min at 20,000*g* refrigerated at +4 °C. The sample was transferred into the LC vial by filtration of the residue through a 0.45 µm syringe PVDF filter.

2.2.2. LC–MS/MS targeted analysis

Chromatography was performed on a Shimadzu LC-20AD-XR system (Kyoto, Japan) fitted with a Phenomenex Kinetex C18 column (100 mm × 2.1 mm, 2.6 µm) and protected with a C18 security guard system from Phenomenex. A gradient was applied and started with 30% B during 1 min. It was then raised to 80% B over 5.5 min, then set to 95% B for 0.1 min and hold for 3.4 min, and again set to 100% B for 0.1 min and hold for 4.4 min. The initial mobile phase composition was

then recovered over 1-min delay. The flow rate was 0.4 mL min^{-1}. The MS equipment consisted of a Sciex API5500 mass spectrometer (San Jose, CA, USA) controlled by the Analyst software (Vers. 1.6.2) operating in positive ion electrospray mode for the confirmation of the dye analytes (2 MRM transitions per analyte). The mass spectrometric conditions are depicted in supplementary data Table S1. The positioning of the source was adjusted; cone voltage and collision energy were tuned to optimize the transition of the precursor ion to the most abundant product ions monitored in the MRM mode. The following MS/MS parameters were used: source temperature, 600 °C; turbo-ion spray voltage, 2000 V; sheath gas pressure (air), 50 psi; auxiliary gas pressure (air), 55 psi; curtain gas, 30 psi.

2.2.3. Validation in aquaculture products

According to Commission Decision No 2002/657/EC (Community, 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, 2002), matrix-fortified calibration samples and matrix-fortified validation samples were selected to estimate the required qualitative and quantitative performance parameters. The adequate levels for fortifying these samples were chosen according to the sensitivity of the method. The lower levels acceptable near the limit of quantification were assessed during the development of the method. The different validation ranges for each of the analytes are described in Table 1. The calibrants consist of samples built with a blank matrix spiked with known concentrations of the analytes of interest. These samples are only used for calibration purposes. They must be prepared according to the protocol that will be further applied for routine checks. One calibrating series with relevant concentration levels including a blank sample and injected twice were replicated on three different days. These 3 × 2 series were considered for the building of the quantitative regression equation. The validation samples are the reconstituted samples with a blank matrix containing known concentrations of the analytes of interest. In the validation process, the validation samples describe the future real-life samples that the analytical procedure will have to quantify. Six replicates were used at each concentration level and for three days, that is to say 18 samples were prepared per level of concentration. Each day of validation was applied for one species (trout, salmon, prawns), and for each species 3 batches from different origins were studied.

The method's performance was assessed for each analyte through both its qualitative parameters such as retention time, specificity, signal/noise ratios, relative ion intensities, and its quantitative parameters such as linearity, accuracy in terms of trueness assessed through the evaluation of recovery and precision, limit of decision CCα.

2.2.4. Implementation of the method on routine samples

Application on aquaculture products purchased from local markets was carried out after the validation of the targeted method. Twenty-eight samples from different species and different origins were monitored: four batches of trout, seven batches of prawns, six batches of salmon, four batches of sea bass, three batches of turbot, one catfish, one tilapia, and one panga fish.

2.3. Metabolomics approach

2.3.1. Animal study and sample collection

The animal study was conducted in order to compare fingerprints to identify a possible metabolic correspondence. In fact, malachite green is the reference triarylmethane dye as it is demonstrated having very effective treatment in fish farming. VPBO was chosen because it has been proven a potential emerging dye in aquaculture (2010.1372 RASFF notification) and it is structurally close to triarylmethanes. So it is interesting to search for common effect biomarkers in case of other similar emerging dyes. Fig. 1 depicts the experimental design of the metabolomics study.

Table 1
Performance parameters obtained during the validation.

Analyte	Specificity (retention time, observation of blanks)	Identification (S/N)	Conclusion(s) SCREENING	Identification (ion ratios)	Validation range (µg kg^{-1})	Trueness (%)	Repeatability RSDr (%)	Intermediate precision RSDrw (%)	Linearity (Mean R^2)	Conclusion(s) CONFIRMATION	Decision limit CCα (µg kg^{-1})
CV with ox	C	C	✓	C	0.1–0.4	94.4–96.6	4.1–6.9	3.9–12.8	**0.9968**	✓	**0.02**
VBB with ox	C	C	✓	C	0.1–0.4	93.3–96.5	4.8–9.1	7.4–13.8	**0.9951**	✓	**0.03**
AZB with ox	C	C	✓	C	0.5–2.0	89.8–91.2	7.6–9.2	7.3–9.1	**0.9814**	✓	**0.27**
VBR with ox	C	C	✓	C	0.1–0.4	96.0–98.6	4.6–8.8	4.2–12.0	**0.9963**	✓	**0.03**
MB with ox	C	C	✓	C	0.5–2.0	87.6–89.7	8.9–9.8	10.2–12.7	**0.9673**	✓	**0.42**
BG with ox	C	C	✓	C	0.1–0.4	99.6–103.5	5.3–9.0	4.8–11.3	**0.9977**	✓	**0.02**
RHB with ox	C	C	✓	C	0.1–0.4	91.5–94.8	7.0–23.0	6.9–21.6	**0.9891**	✓	**0.04**
MG with ox	C	C	✓	C	0.1–0.4	91.8–93.6	15.7–19.2	22.8–26.5	**0.9914**	✓	**0.04**
EV with ox	C	C	✓	NC	0.1–0.4	79.2–88.5	12.0–16.1	14.2–17.7	**0.8879**	×	**0.15**
NBA with ox	C	C	✓	C	0.1–0.4	95.2–96.9	11.1–12.9	14.9–18.0	**0.9909**	✓	**0.04**
RHG with ox	C	C	✓	C	0.1–0.4	96.8–104.7	6.8–24.3	10.3–26.4	**0.9758**	✓	**0.06**
NMB without ox	C	C	✓	C	0.5–2.0	94.8–96.9	4.4–6.0	5.5–7.4	**0.9949**	✓	**0.14**
PRRA without ox	C	C	✓	C	0.1–0.4	92.7–96.0	6.8–8.6	7.2–9.8	**0.9871**	✓	**0.04**
VPBO without ox	C	C	✓	C	0.1–0.4	90.7–95.2	7.7–8.7	7.4–15.8	**0.9850**	✓	**0.06**

R^2 = determination coefficient.

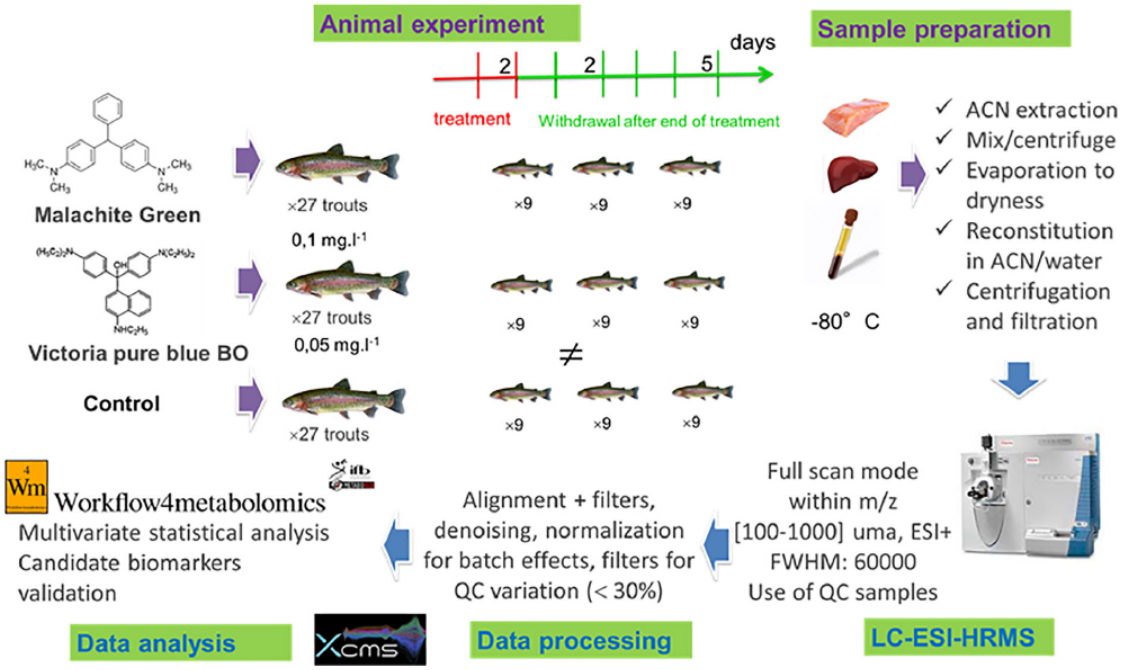


Fig. 1. Experimental design of the metabolomics study.

The objective of the animal study was based on two experimental procedures. During a pilot phase, the toxic effects of malachite green (MG) and of victoria pure blue BO (VPBO) on trouts were evaluated. In the literature some dose of administration for malachite green were suggested (Bajc, Jenčič, & Šinigoj Gačnik, 2011) however no data were found for the administration of victoria pure blue BO in aquaculture. The objective of the second phase was finally to search for metabolic fingerprints following administration of the two dyes on separate groups of trouts.

2.3.2. Fish

A total of 81 specific pathogen-free rainbow trouts composed of a mixture of males and females from the same genetic group were used for this experiment. The 81 rainbow trouts used for this experiment were sourced from virus-free fish rearing facilities of the ANSES Plouzané laboratory site (France). This batch of fishes was one year old and healthy specimens at the beginning of the project (105 ± 25 g and 15 ± 2 cm). Before the start of the experiments, some individuals were subjected to autopsies as well as to bacteriological and virological analyses to check their health status. They were fed with commercial dry pellets at 1.5 percent body weight (Neo Prima 4, Le Gouessant Aquaculture) once a day.

2.3.3. Authorization to experiment and ethical aspects

This experiment was conducted in accordance with European Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. The ANSES site of Plouzané holds the authorization to conduct experimentations on fish in its facilities according to the administrative order no. C29-212-3 delivered by the Prefecture of "Département du Finistère". Furthermore, the project in which this work took place was evaluated for a specific authorization issued by the French Ethics Committee Comité Français d'Ethique).

2.3.4. Experimental system

The experimental system consisted of six similar independent cylindrical tanks (75 L) in an open circuit with purified fresh river water at a flow rate of 0.3 $m^3 h^{-1}$. This water inlet maintained optimal water conditions for the fish (temperature of 13 ± 1 °C, dissolved oxygen < 90%, pH close to 8 and free of nitrate and nitrite) throughout the experiment. Tanks were maintained in a natural light/dark cycle (14 h/10 h in spring approximately) in a room with an air volume change every hour. Water temperature was continuously measured and recorded by means of a wireless probe (Cobalt, Oceasoft®) coupled to an acquisition system (ThermoClient 4.1.0.24). Oxygen saturation was monitored using Oxymeter WTW-OXI315I.

2.3.5. Exposure conditions and depuration period

Firstly, the 81 fishes were randomly distributed in three tanks of the experimental system. After one week in acclimation, the system was positioned in a closed-circuit system and one tank was exposed to 0.10 mg L^{-1} of MG, the second was exposed to 0.05 mg L^{-1} of VPBO, while the third was let free of dye exposition for control (same experimental procedures but without pollutant). After a 48-h bath, the fishes were transferred in three new tanks, for a five-day depuration period in a purified fresh river water open circuit.

2.3.6. Samples and sampling dates

Nine fishes were randomly sampled from each tank at three sampling dates, after the 48-hour exposure period, 30 h after the beginning of the depuration period and at the end of the five-day depuration period. For each fish, 2 mL of blood was withdrawn from the caudal vein by means of a lithium heparinized vacutainer (BD Vacutainer LH 85 IU). Whole blood samples were centrifuged (1200 g, 10 min, 4 °C) and plasma was stored at −80 °C. Fishes were killed after collecting blood by percussive stunning with a head blow and were then measured and weighed. Liver and muscles were removed and stored at −80 °C not more than two months for future analysis.

2.3.7. Sample preparation for plasma, liver, muscle

The sample was weighed (2 g of mixed muscle, 500 µL for plasma and 900 mg of mixed liver) into a centrifuge tube. Then 50 µL of the internal standard solution (MG-d_5 and CV-d_6 at 100 µg L^{-1} in acetonitrile) was added. The sample was vortex-mixed and let to stand for

10 min in a dark place. Then acetonitrile was added (8 mL for muscle, 4 mL for plasma) or a mixture of methanol/acetonitrile (50/50, v/v) for liver. The sample was vortex-mixed to homogenize the material with the solvent. The sample was further placed on a mechanical rotary shaker for 10 min at 100 rpm and centrifuged for 5 min at 4000*g* refrigerated at 4 °C. The supernatant was transferred to a polypropylene tube (5 mL for muscle, 4 mL for plasma and liver) and evaporated to dryness under gentle nitrogen flow at 50 °C. The residue was dissolved in acetonitrile/water for reconstitution (80/20, v/v) (600 µL for muscle, 400 µL for plasma and liver), vortex-mixed and then centrifuged for 5 min at 20,000*g* refrigerated at 4 °C. The sample was transferred into the LC vial by filtration of the residue through a 0.45 µm syringe PVDF filter. A pooled quality-control (QC) sample for each matrix was prepared by combining 5 µL of each extract from each sample.

2.3.8. LC-HRMS analysis

Chromatography was performed on a Thermofisher U-HPLC Accela system (Bremen, Germany) fitted with a Waters Xbridge BEH C18 column (100 mm × 2.1 mm, 2.5 µm) and protected with a C18 security guard system from Phenomenex. A gradient was applied and started with 2% B. It was then raised to 98% B over 13 min and hold for 4 min. The initial composition was then recovered over a 1-min delay. The flow rate was 0.30 mL min^{-1}. The MS equipment consisted of a LTQ Orbitrap XL MS mass spectrometer (Thermofisher, Bremen, Germany) controlled by the Xcalibur software (Vers. 1.6.0) operating in positive ion electrospray mode of the analytes. The positioning of the source was adjusted to obtain the optimal signals. The following parameters were used: capillary temperature, 275 °C; turbo-ion spray voltage, 5000 V; sheath gas pressure (air), 30 (arbitrary unit); auxiliary gas pressure (air), 10 (arbitrary unit); sweep gas, 2 (arbitrary unit). The instrument was calibrated to reach mass accuracies in the 1–3 ppm range. It was operated in full-scan FTMS over *m/z* range of 100–1000 Da at a resolving power of 60,000 (full width at half maximum). For each matrix, LC-HRMS samples acquisitions were randomized and quality-control (QC) samples for each batch, were injected every 5 samples in the sequences.

2.3.9. Data processing and statistical analysis

The raw (*.raw) LC-HRMS files were converted to a more exchangeable format (*.mzXML) using MSConvert. The data were then processed using both R software (xcms 1.44) and the collaborative Galaxy platform "Workflow4metabolomics" version 3.0 (Giacomoni et al., 2015). Workflow4metabolomics, a collaborative research infrastructure for computational metabolomics, is a collaborative tool for comprehensive metabolomics data pre-processing, statistical analysis, and annotation (Giacomoni et al., 2015; Guitton et al., 2017).

Because there were numerous satellite peaks as artifacts from Fourier Transform calculations, the converted MS data files were firstly filtered by eliminating all *m/z* signals higher than 750 Da. This filtration was made with in-house R scripts to improve quality of data. Then the overall workflow of data processing consisted in as follows: (1) the peak extraction (xcms R package) (Smith, Want, O'Maille, Abagyan, & Siuzdak, 2006), (2) the peak alignment (xcms R package), (3) the filtering of *m/z* within the range of [45–1020] seconds by eliminated all non-gaussian profiles, (4) the elimination of the colinearity between ions, more precisely the filtration of isotopic ions or adducts after annotation with the CAMERA package (Kuhl, Tautenhahn, Böttcher, Larson, & Neumann, 2012), (5) the normalization in order to correct the signal drift and batch-effects by fitting a locally weighted regression model (Lowess) to the QC values (Van Der Kloet, Bobeldijk, Verheij, & Jellema, 2009), (6) the elimination of ions having a coefficient of variation (CV) of signal intensity higher than 30% in QC pool sample.

The parameter settings used for peak detection and alignment were optimized using the R-package IPO ('Isotopologue Parameter Optimization') (Libiseller et al., 2015).

The optimal parameter settings which were used for the overall process of data are presented in supplementary data Table S2.

Before normalization, a table matrix containing the intensity areas for each feature defined by the couple retention time and *m/z* was generated. Then, after normalization and the different filtration steps, the refined matrix was used for statistical analysis of data. Statistical analysis was performed by using the implemented tools on Workflow4metabolomics (Giacomoni et al., 2015) and Simca v. 14.0.0.1359 (Umetrics AB).

The most commonly used unsupervised multivariate analysis, i.e. Principal Component Analysis (PCA), was firstly performed for each matrix (muscle, liver and plasma) in order to visualize any patterns and groupings within the data without considering the belonging of the samples to a group ('Control' or 'Treated' group). PCA technique consists in the reduction of the dimensionality of a dataset containing a higher number of variables than number of samples, and transformation into few orthogonal latent variables called principal component (PC). In that way, the PC representing the greatest variability in the dataset is susceptible to give information about the origin of the variability, i.e. whether or not it is assigned to the treatment with dyes. Once Log_{10}-transformed and Pareto scaled, data were submitted to PCA analysis with the algorithm svd.

Supervised partial least square discriminant analysis (PLS-DA) were then conducted in order to find the features (ions) causing the highest discrimination between the groups of samples ('Control' versus 'Treated' group). These potential markers of treatment with dye were selected according to the criterion named "Variable of Importance in the Projection" (VIP). VIP criterion is a measure of the contribution of the feature (variable) in the model besides its importance in the prediction of the response variable (i.e. the group of the sample). Similar to PCA modelling, data were Log_{10}-transformed and pareto scaled before PLS-DA which was proceeding under the algorithm nipals. Diagnostic statistics were also carried out in order to check the quality and performances of the constructed model (R^2X, R^2Y, Q^2Y, the permutation diagnosis, the detection of outliers by scoring, and cross validation performances).

Univariate analyses were also performed to determine which features differed significantly from 'Control' to 'Treated' group according to their signal intensity. In that aim, Student's tests and non-parametric tests of Wilcoxon were both carried out with a Bonferroni correction and a p-value threshold set at 0.05.

The ultimate selection of markers of treatment was made by the gathering of all results obtained from all multivariate and univariate analyses, and the comparison between all these results. At last, the analytical qualities of the selected markers were evaluated through the examination of their respective extracted ion chromatograms (EICs).

Two procedures were used for the identification of potential biomarkers. First, direct metabolite formation investigations were made using MetWorks 1.3.0. SP1. Software (Thermo Fisher Scientific, Waltham, MA, USA). The extraction's mass window was set at 5 ppm. Tentative identification of the endogenous markers was then carried out by using three different databases: Chemspider, KEGG, HMDB, and assigning possible adducts with K, NH_4, Na, H with accounting an experimental error lower than ± 5 ppm.

3. Results and discussion

3.1. Targeted LC–MS/MS approach

3.1.1. Sample preparation

According to Annex I of Directive (EC) No 96/23 for the substances to be controlled in foodstuffs of animal origin, the pharmacologically active dyes belong to the Group B3e. Currently, only the malachite green has been set a RPA of 2 µg kg^{-1} for the sum of malachite green and its leuco-base metabolite (Contam- Malachite (Scientific opinion), 2016).

In France, the current official method for controlling residues of

dyes of interest monitors three compounds: the malachite green, the crystal violet and the brilliant green. The occurrence of the malachite green residues in food from aquaculture origin tends to decrease over the 15 past years worldwide when other dyes are now disclosed in the literature as potential zootechnical agents. During our project, a targeted and very sensitive method was developed and validated to extend the scope of investigation of these compounds. Regarding the number of dyes included in the mass spectrometric methods found in the current literature, a first evolution was to include the crystal violet and the brilliant green in the analytical control (Andersen et al., 2009; Doerge, Churchwell, Gehring, Pu, & Plakas, 1998; Hurtaud-Pessel et al., 2011). More recently, multi-class methods for control of dyes in seafood appeared over the last ten years (Reyns et al., 2014; Sun, Sun, Li, Zhang, & Yang, 2013; Tarbin et al., 2008; Xu et al., 2012).

Derived from the methods of Tarbin et al. and of Reyns et al., the present method aims at confirming and quantifying by LC–MS/MS the residues of 14 dyes in aquaculture products. The dyes chosen to be included were similar to those in the Tarbin and Reyns methods, i.e. arising from five families, as our review from the literature showed that no other potential pharmacologically active dyes are currently depicted. For the chromatographic separation, a compromise between UHPLC and classical HPLC separation was defined. A 2.7 µm analytical column conditioned in a 14-min run gave efficient separations especially from interferences brought by fat fishes such as salmon or trout. The target tissues for this study were: 1 – trout tissues because of this major finfish freshwater farming production in France, and 2 – salmon and prawns because of their high rate of import and European consumption. Some of these dyes are already known to be rapidly metabolized in their reduced leuco form and most of the analytical methods described in the literature target the known leuco-bases (leuco malachite green, leuco crystal violet). However, potentially existing leuco forms of other dyes may have to be monitored as well and all the relevant chemical standards are not necessarily commercially available. This is the case for instance for the leuco brilliant green already described by Hurtaud-Pessel et al. (2011). The analytical method we have developed in line with some other similar methods from the literature, aims at oxidizing the leuco metabolites to finally dosing the only parent molecules instead. The applied oxidation originates from the Roybal et al. extraction (Andersen, Turnipseed, & Roybal, 2006; Turnipseed, Andersen, & Roybal, 2005) who proposed to add the oxidant, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), directly during the extraction step. This improvement brought enough sensitivity to convert the leuco malachite green into malachite green and DDQ was adopted by some other authors (Reyns et al., 2014; Tarbin et al., 2008). During the development of our standard operating procedure, the optimal amount of DDQ was set at a volume of 2 mL at 5 mM, a lower volume conducted to incomplete oxidation in our case. Besides it was noticed that some compounds, pararosalinine, new methylene blue, and victoria pure blue BO, undergo a loss at above 50% caused by oxidation and also show an alteration of the peaks. So it was undertaken to validate the method in two parts, one with oxidation and one without oxidation, and to collect and compare the validation data. The extraction was performed with 6 mL of acetonitrile and addition of anhydric $MgSO_4$ for 2 g of matrix. No purification by solid phase extraction was set up as performance and quantitation were satisfying during the process of development. Moreover, after the total evaporation of the extract, the volume of reconstitution solution added to the extract (600 µL) was found enough to avoid troublesome matrix effects. The choice was made to reconstitute the samples with a mixed solution of acetonitrile and of ascorbic acid to stabilize the parent forms once they are converted.

For the validation without oxidation, a small fraction of 300 µL was removed from the extract just before the oxidation step and sent to LC–MS/MS directly after filtration.

3.1.2. Validation

The validation was performed in accordance with criteria of performance from the Commission Decision (EC) No 2002/657. Matrix-fortified calibration standards (SC) and matrix-fortified validation samples (SV) were selected to estimate the required qualitative and quantitative performance parameters to be validated (Dubreil-Chéneau, Pirotais, Verdon, & Hurtaud-Pessel, 2014). The adequate levels for fortifying the SC and SV samples were chosen following the sensitivity of the dyes and then separated in two level groups. Each day of validation was devoted to one species (trout, salmon, prawns) and four different origins were used per species, one for the calibration curve and three other origins for the SVs. All validation parameters, e.g., the decision limit CCα, the detection capability CCβ, the precision, and the recovery were evaluated. The levels for SC and SV ranged between 0.1 and 0.4 $\mu g\,kg^{-1}$ for eleven compounds and between 0.5 and 2.0 $\mu g\,kg^{-1}$ for three compounds AZB, MB, NMB (see all results in Table 1).

Identification criteria. Pharmacologically active dyes are non-authorized substances in seafood-producing species. So four identification points are necessarily earned by monitoring two MRM transitions for each of the 14 analytes to detect each of them unequivocally. The specificity of the method was tested for the two MRM transitions of each analyte by comparing three types of chromatograms obtained from standard solutions, from different blank samples and from spiked matrices, respectively. No interference was observed at the retention times of any of the 14 dyes and of their internal standards. Fig. 2 shows a chromatogram of a spiked trout muscle sample at the first level of quantification.

The ion ratios corresponding to the least intense versus the most intense signal, and the signal-to-noise ratios were calculated for each analyte on the SC and SV. The relative deviations of the ion ratios according to Decision (EC) 2002/657 were satisfactory for all analytes (see supplementary data Table S1). The signal-to-noise ratios were also all satisfactory, as indicated in Table 1, with values far greater than three.

Quantitative criteria. Response linearity was determined using a linear regression model. One matrix-fortified calibration curve was built each day with four calibration levels in concentration ranging between 0.1 and 0.4 $\mu g\,kg^{-1}$ for eleven compounds and between 0.5 and 2.0 $\mu g\,kg^{-1}$ for the three compounds AZB, MB, NMB. Linearity was successfully achieved over the calibration range of the method with R^2 above 0.96 for all the analytes except EV. EV did not reach a sufficient linearity (mean R^2 of 0.8879) because a stability decrease was observed during the batch analysis. A decrease by two of the area between the first calibration curve and the second calibration curve precluded to fully validate the method for EV. This issue should be further investigated in near future. For other compounds, calibrating curves were all considered linear, with coefficients of determination (R^2) over 0.9673 for MB. Trueness was assessed through the recovery calculated from the matrix-fortified calibration curve created each day by back-calculating the SV concentrations at each level of concentration. The percentage of recovery was given by the mean of the back-calculated concentrations for each level of concentration divided by the theoretical spiked concentration. Table 1 displays the recovery results in terms of trueness. Commission Decision (EC) No 2002/657 recommends trueness between 50% and 120% for concentrations below 1 $\mu g\,kg^{-1}$, and trueness between 70% and 110% for concentrations between 1 and 10 $\mu g\,kg^{-1}$. Therefore, all results fulfilled the recommendations. For the 11 dyes detected after oxidation, the values ranged from 79.2% for EV to 104.7% for RHG. For the three compounds validated without oxidation, i.e. new methylene blue, pararosaniline, and victoria pure blue BO, respectively, results ranged from 90.7% to 96.9%. The precision of the method was evaluated at each SV level of concentration by

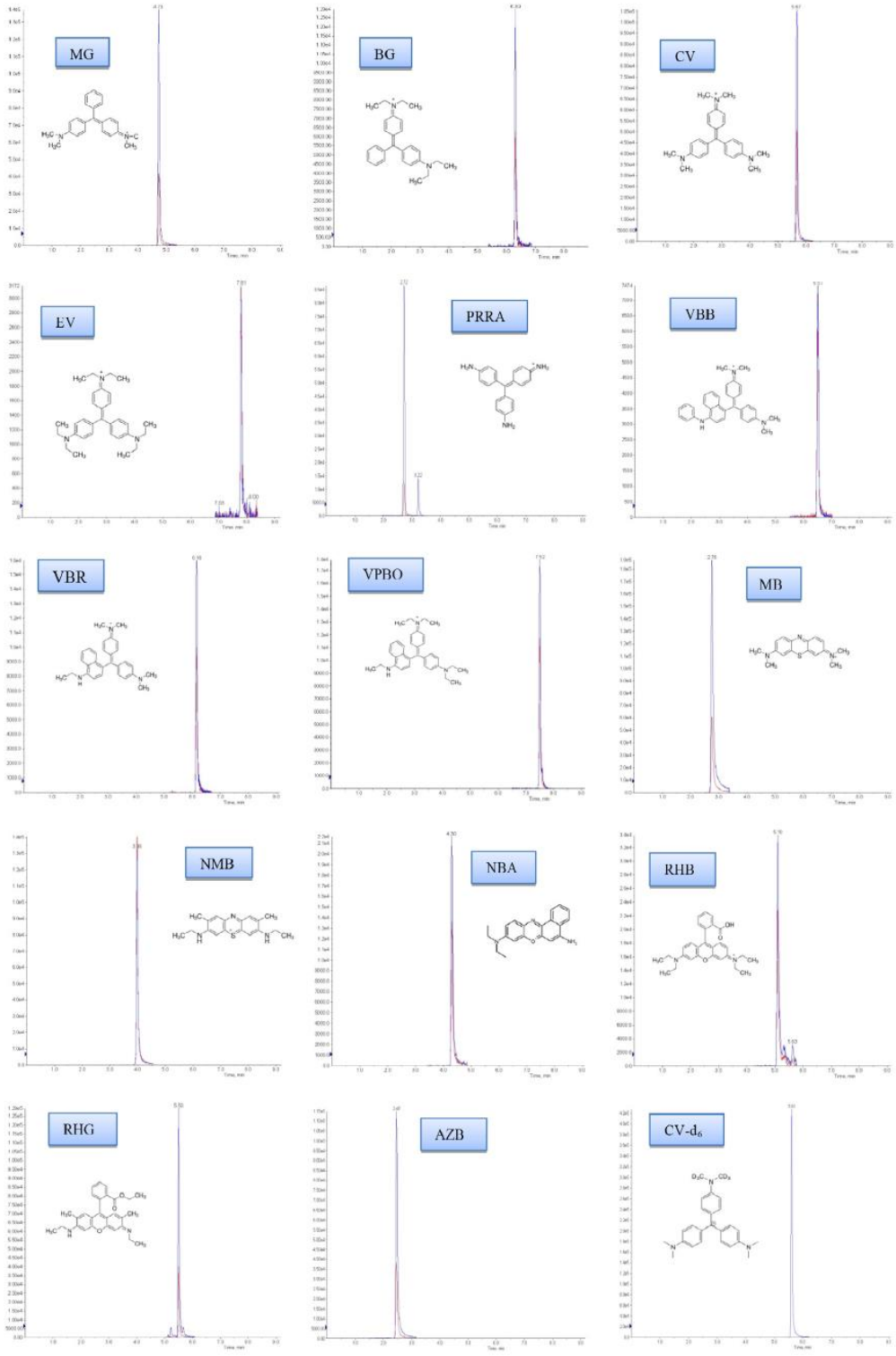


Fig. 2. Extracted MS/MS chromatograms of all dyes spiked in trout tissue at concentration at level 1.

calculating the relative standard deviation (RSD%) in repeatability conditions (RSD calculated from the six replicate SVs of the day) and in intra-laboratory reproducibility conditions (RSD calculated from the 18 replicate SVs over three days). Table 1 gives the results of repeatability (RSDr%) and intermediate precision (intra-laboratory reproducibility, RSDrw%) tests. The highest RSD obtained for precision were 23.0% for RHB and 24.3% for RHG, and 26.5% for MG and 26.4% for RHG for intermediate precision. The RSDs for concentrations lower than

100 μg kg^{-1} shall be as low as possible according Commission Decision No 2002/657/EC. We consider that 30% for RSDr and RSDrw is an acceptable limit for the coefficient of variation considering this very low range of concentration, which is the case for results for all validation concentrations.

The critical limit CCα was determined in accordance with the so-called calibration curve procedure of the ISO Standard 11843 and considering the case of the linear regression, as proposed in European Decision N° 2002/657/EC. The highest CCα obtained for the 11 compounds in the validated range of 0.1–0.4 μg kg^{-1} was 0.06 μg kg^{-1} for RHG. For the 3 compounds in the validation range of 0.5–2.0 μg kg^{-1}, the highest CCα obtained was 0.42 μg kg^{-1} for MB. It is generally accepted the maximum CCα for a non-authorized substance shall tie at least to the first level of calibration to be chosen below the regulatory RPA. All our results fulfill this requirement except for EV because of excessive variation in the second injection of the calibration curve.

Based on the quantitative criteria of the Commission Decision (EC) No 2002/657, all the quantitative technical performance criteria are met for all the 14 dyes except for the ethyl violet (EV). For the confirmatory identification purpose of the analytical method and over the 14-targeted dyes included, 10 of them were fully validated from those with the oxidation step, and also the 3 dyes without the oxidation step were fully validated as well. The levels achieved for the decision limits were all assessed below 0.5 μg kg^{-1}, that are far below the RPA proposed at 2 μg kg^{-1}. For screening, the method is fit for purpose for the 14 dyes. The method is also fully validated for different aquaculture species. Inter and intra-species variability was additionally included in the validation results. In conclusion the LC–MS/MS targeted method is applicable for confirmation in aquaculture products, for the following dyes:

- with oxidation: crystal violet, victoria blue B, azure B, victoria blue R, methylene blue, brilliant green, rhodamine B, malachite green, ethyl violet, Nile blue A, rhodamine 6G
- without oxidation: new methylene blue, pararosaniline, victoria pure blue BO

3.1.3. Analysis of marketed aquaculture products

An experimental plan specifically dedicated to this project study was carried out using the targeted method for the 28 samples (8 species) collected from local markets. Two samples were suspected containing dyes (MG, VPBO) at very low concentrations, and below the first level of calibration. An estimated concentration at 0.036 μg kg^{-1} of MG in a seabass and an estimated concentration at 0.066 μg kg^{-1} of VPBO in Tilapia were noticed. Even identification criteria were fulfilled, the estimated concentrations were far below the first concentration of calibrants at 0.1 μg kg^{-1} for theses dyes. For this reason, only screening was implemented and confirmatory step of these two samples in duplicate was not initiated.

3.2. Metabolomics LC-HRMS approach

3.2.1. Metabolomics study conditions

The animal experiment involved homogeneous animal population in terms of age, breed and nutrition. Breeding is the first key step to control in a metabolomics study. Such studies have been poorly applied to farmed fish and particularly for trout exposed to chemical contaminants (Samuelsson, Björlenius, Förlin, & Larsson, 2011). To our knowledge, only De Sotto et al. (2016) reported a metabolomics study in fish (zebrafish) applied to pharmacologically active substances, a sulfonamide.

The item of our study was one year old juvenile trout. At this stage, the immune and antioxidant capacities are mature but not yet interfering with reproduction. Rainbow trout has long been a model species in toxicology. Moreover, trout fish farming has a very strong economic importance in France. It appears to be relevant for our study and under full control in our fish experimentation facilities. To ensure adequate breeding conditions it was carried out several actions: observation of the living behavior, removal of the dead, emptying of the water bath and cleaning, food (ad libitum), control of the temperature of the water and of the main physicochemical parameters. The administration dose fixed after the pre-toxicity study was 0.10 mg L^{-1} in a controlled water bath during 2 days for MG and 0.05 mg L^{-1} in a controlled water bath during 2 days for VPBO. Despite all precautions, we observed the death of sevens trout during VPBO treatment due to a technical issue with the air intake. So a batch of five trouts were kept for the 30 h depuration period and 6 for the five day depuration period instead of nine each. During the study, a control group was bred simultaneously and same number of control trout was taken simultaneously as for treated trout. Only one death was observed in the controls. When sampling, the biological matrices (liver, muscle, and plasma) were stored immediately at −80 °C to avoid any alteration. For analysis of samples, it was made typical choices in terms of extraction and analysis by LC-HRMS. Metabolomics analyses presume that the analyst will spend a lot of time on data treatment, and in this case for the three selected matrices. So decision was taken to perform only one extraction condition per matrix (acetonitrile or methanol extractions) and to analyse samples in ESI positive polarity only. In fact, methanol and acetonitrile are generic solvents most often able to extract molecules in a wide polarity range. ESI positive polarity was chosen because it allows to correctly detect the positively charged triarylmethanes and their major potential metabolites as well as theoretically a larger number of endogenous metabolites (Knolhoff, Kneapler, & Croley, 2019).

The data acquired by LC-HRMS in full scan for the «treated trout from each dye group: the MG group and the VPBO group (VB-called on PLS-DA graphs)» were compared together to the «group of non-treated trout or control group», by operating statistical data treatments on the generated datasets. The repeatability and quality of data acquisition was checked by using two isotope labeled internal standards and by injection of quality-control (QC) samples for each batch. A correct alignment of extracted peaks was obtained for peaks below 750 Da. The method was optimized in order to get fingerprinting of samples so no quantification of MG and VPBO and no CID experiment were additionally performed. Peak area of MG and VPBO were collected for plasma, muscle and liver at the different times of treatment in order to assess trends of depletion of parent compounds. The surface time profiles of MG and VPBO in the different matrices during treatment and after treatment are presented in Fig. 3. The MG and VPBO behavior were similar in the different matrices with a slight decrease in plasma and muscle and a stronger decrease in liver at 4 days after withdrawal of treatment. The depuration period of 4 days was not sufficient to eliminate completely the parent dyes. This is in compliance with literature where MG as its colored form without measurement of LMG is displayed to be persistent up to 60 days after a 6 days long bath exposure (Máchová et al., 1996).

3.2.2. Multivariate statistical fingerprinting

The Galaxy platform permitted to obtain a metabolic fingerprinting of treated trout for two dyes, ie. MG and VPBO, compared to a control group. Our first objective was to conduct the data treatment for treated trout either by MG or VPBO but not to separate the two treatments to compare to controls in order to collect similar effects on the two dyes.

The data were processed for the three sets of matrices but are detailed here only for muscle and liver. In fact, data processing for plasmas did not lead to significant models. Nevertheless, PLS-DA for plasma is included in supplementary data Fig. S1. After a first preprocessing, the retention time alignment of all samples was not satisfactory due to a dense chromatographic region between 600 and 900 s. Change of the column type did not improve the quality of chromatograms. RT alignment was however improved by removing the masses higher than 750 Da. The data global processing detailed in Section 2.3.4 led to the selection of 422 features for muscle and 659

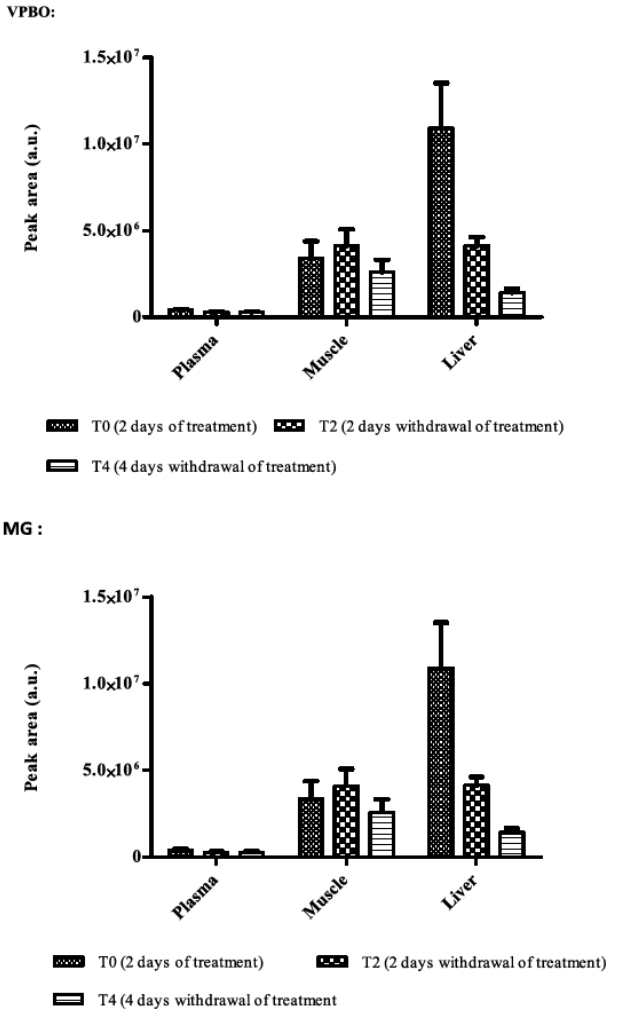


Fig. 3. Surface time profile of MG and VPBO in the different matrices during treatment and after end of treatment.

features for liver. Principal-components analysis (PCA) was first used to exploit the global dataset. The PCA carried out (see supplementary data Fig. S2), after correction of the batch effects and after the application of the filters on the ions, did not make it possible to discriminate the treated fish from the controls, whatever the matrix. On the other hand, the PCA confirmed that the corrections to normalize the batch effects made it possible to gather the QCs and, by extension, to state on the values of the conditions. The PCA released also the detection of intrinsic clusters or outliers. For muscle, the analysis revealed six outliers, three for malachite green and three for victoria pure blue BO. Partial least squares discriminant analysis (PLS-DA) was then used to model the relationship between exposure conditions and LC-HRMS data. An initial model for each matrix was built on the basis on the global data set retained, the 422 features for muscle and the 659 features for liver. The models (Fig. 4) for muscle (Fig. 4a) and liver (Fig. 4b) clearly differentiate between group of controls and group of treated animals, with satisfactory descriptive and predictive values, for muscle: R^2X (cum) = 27.7%, R^2Y (cum) = 91.9%, Q^2Y (cum) = 82.8, and for liver: R^2X (cum) = 32.2%, R^2Y (cum) = 98.4%, Q^2Y (cum) = 91.2%. Although the group of animals treated with MG or VPBO were differentiated from the group of controls, no strict separation was observed between MG treatment and VPBO treatment, which could mean that similar effects could occurred on trout by the two dyes. For muscle, the model, after elimination of correlated ions with reviewing extracted ion chromatograms (EICs), revealed 11 features significantly different among the 3 groups, with a VIP value greater than 2.0.

Final PLS-DA of the muscle reduced data sets resulted in a model with good descriptive value (R^2X = 60.2%) and good prediction (Q^2Y = 83.1%) with a high degree of explained variation (a total R^2Y = 86.4%) modeled for the first 2 variables. For liver, a total of 17 ions significantly different between the treated group and the control group were selected to build the final model with satisfactory values: R^2X (cum) = 41.3%, R^2Y (cum) = 79.2%, Q^2Y(cum) = 76.4%. The graphs of final PLS-DA are presented in the supplementary data Fig. S3. The robustness of the models was assessed by using permutation tests and cross validation. Results from these tests were all satisfactory (data not shown), with 0% of false negatives for the cross validation. These validated models prove that high confidence can be accepted in the features retained as potential biomarkers of treatment. Nevertheless, it would be necessary to validate definitely the models by novel data sets of treated trout.

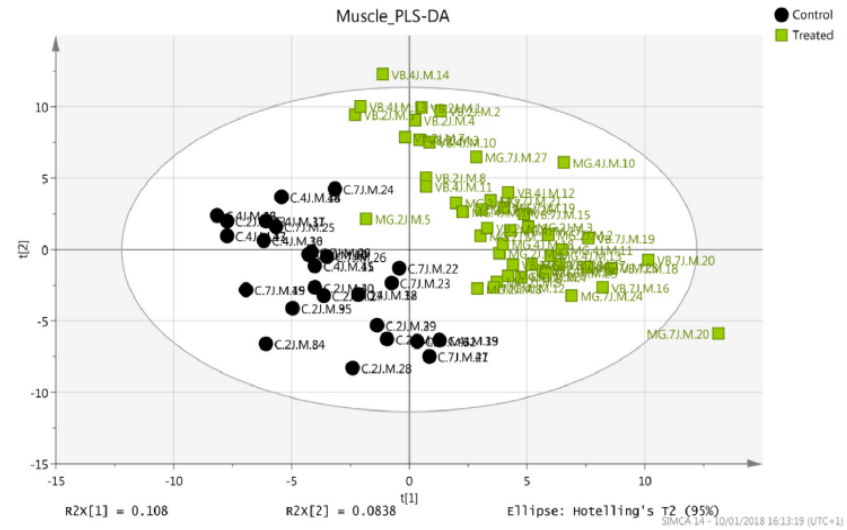
3.2.3. Potential biomarkers

The features selected according to the various criteria (final model, VIP values, EICs.) resulted in a list of potential biomarkers that were specifically examined. Firstly, in order to try to identify MG or VPBO related metabolites, probable phase I and phase II most common biotransformations pathways were studied. The formation of metabolites was investigated using MetWorks software. Starting from the parent compound mass (m/z = 329.2012 for MG and m/z = 478.3216 for VPBO), we screened a wide panel of Phase I and Phase II reactions based on mass shifts. The exact molecular masses of the corresponding molecular ions were compared with the experimental m/z values of the retained features to try to confirm the identity. For other potential biomarkers, tentative identification in the different databases assigning positive charge (molecular ion and probable adduct ions) and an experimental error lower than 5 ppm was carried out to find out probable elemental composition and associate mass error.

Most of retained features were in fact direct metabolites of MG and VPBO, and especially for the liver group. No common potential biomarkers were found sufficiently relevant between MG and VPBO treatment in the muscle group. In liver, some features could be common as a response of trout organisms after both treatments, but these ions are currently not characterized.

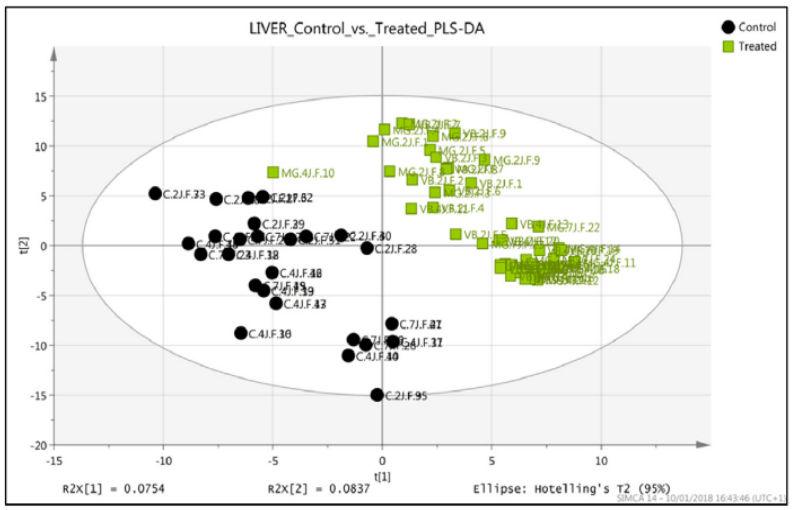
In muscle, as shown in Table 2a, more potential biomarkers are kept for MG than for VPBO, that suggest a greater effect on trout metabolism due to the MG treatment than to the VPBO treatment, in particular for down-regulated endogenous compounds. As an example, the surface time profile (supplementary data Fig. S4) of ion M533T401 reveals its impact after MG treatment over time compared to the control trout. Two direct major metabolites, LMG and *N*-desmethyl LMG that have already been identified a long time ago (Cha, Doerge, & Cerniglia, 2001), were also identified by the software. Other features could not be associated to putative compounds estimated by in silico programs except the feature M517T443 found in three databases. It is proposed as Taurodeoxycholic acid, a bile acid that appears to be affected by MG treatment being down regulated compared to the control group or to the VPBO treatment conditions. The proposed identification was confirmed thanks to the isotopic pattern (presence of one sulfur) and to the injection of a commercial standard solution of Taurodeoxycholic acid. The primary bile acids are synthesized in the liver from cholesterol. Then they are conjugated to either taurine or glycine. Altered bile acid pattern was shown to provide a rich source of biomarkers for early detection of specific liver injuries. Some bile acids were depicted as excellent biomarkers to detect illegal use of nitroimidazoles in pigs (Arias et al., 2016). In fish, especially zebrafish, the primary bile acid pathway was shown as affected by fipronil (Yan et al., 2016). Exposure of fish to wastewater effluent resulted in increases in plasma concentrations of xenometabolites as taurocholic acid (Al-Salhi, Abdul-

(a)



$R^2Y(cum) = 91.9\ \%$ $Q^2Y(cum) = 82.8\ \%$

(b)



$R^2Y(cum) = 98.4\ \%$ $Q^2Y(cum) = 91.2\ \%$

Fig. 4. PLS-DA score plot of metabolites profiles from samples belonging to control and treated (MG and VPBO) trout, for muscle (a) and for liver (b).

Sada, Lange, Tyler, & Hill, 2012). In this study, the down-regulation of the bile acid is induced by MG only. This was confirmed in our study, as shown in liver group of samples, and as displayed in supplementary data Fig. S5 (liver M517T443), where the bile acid was found but was not retained by the model, being probably excluded by a variation of the ion higher than 30% in the samples.

For VPBO in muscle, four ions, including two metabolites were retained as potential markers. Unfortunately no commercial standard solution for VPBO metabolites is available. But metabolites could be effectively confirmed by similar fragment ion source (347.1952 and 362.3658) between parent VPBO and the different metabolites. Deethylations of VPBO are not yet defined in the literature but dealkylations of some other triarylmethanes are known for a long time. The two endogenous compounds found as potential markers were down regulated by VPBO, as shown supplementary in supplementary data Fig. S4 for ion M506T837, but contrary to MG down-regulated ions, these two compounds, still unidentified, seem less polar than VPBO metabolites. Moreover the effect of treatment by VPBO is not lasting among the treatment period. After five days of treatment withdrawal, the peak area of M506T837 has a tendency to return to the basal level.

In liver, as shown in Table 2b, we firstly speculated possible metabolites according to the most common biotransformation pathways, the structure of the parent drug and similarly structured drugs, and possible metabolites were assumed for MG and VPBO, in particular a whole series of metabolites undergoing successive dealkylations. One doubt persists on M389T523 for MG that could be a glycine conjugate but mass error is high. However it should be a direct metabolite of MG because it was totally absent in the controls. For VPBO selected marker candidates, the leuco form was found whereas not found in muscle. Also following VPBO treatment, another bile acid, Glycodeoxycholate, was

Table 2a
Potential biomarkers in muscle between control trout and VPBO or MG treated trout.

MG muscle						
Feature	Trend regulation	*m/z*	Rt (min)	Formula	Mass error (ppm)	Putative metabolite
M331T764	up	331.2163	12.73	$C_{23}H_{26}N_2$	1.67	LMG
M317T687	up	317.2006	11.45	$C_{22}H_{24}N_2$	1.69	*N*-Desmethyl LMG
M434T653	up	434.2852	10.88	/	/	/
M517T443	down	517.3297	7.37	$C_{26}H_{45}NO_6S$	−1.50	Taurodeoxycholic acid (499.296753 + NH_3)
M560T401	down	560.2619	6.68	/	/	/
M533T401	down	533.3245	6.67	/	/	/
M531T395	down	531.3089	6.58	/	/	/
VPBO muscle						
Feature	Trend regulation	*m/z*	Rt (min)	Formula	Mass error (ppm)	Putative metabolite
M452T719	up	452.3052	11.18	$C_{31}H_{37}N_3$	1.92	*N*-Desethyl LVPBO
M393T548	up	393.2853	9.12	$C_{27}H_{27}N_3$	−1.66	*N*-Tri-desethyl VPBO
M506T837	down	506.4196	13.95	/	/	/
M396T674	down	396.3313	11.22	/	/	/

characterised for the M449T545 ion, and confirmed by a standard commercial solution by retention time and exact mass verification. Contrary to MG up-regulation on Taurodeoxycholic acid, VPBO effect seems to increase the level of Glycodeoxycholate. That means that the two triarylmethanes could act differently on the bile acid metabolism, and should presume to different action modes of the pharmacologically active drugs.

However, in the liver group three endogenous markers are assumed to be representative as a common action mode between MG and VPBO. They undergo up or down trend regulation by both MG or VPBO. As presented in supplementary data Fig. S4, the surface time profile of ion M717T443 is not marked clearly between treated MG or VPBO trout on the one hand and control trout on the other hand. These ions are still not identified, and could represent possible common relevant markers for forbidden triarylmethanes treatment, unfortunately their intensities in this study are rather weak.

4. Conclusion on interest of targeted and metabolomics approaches for dyes

In this overall study, we firstly developed and validated a targeted method in aquaculture products allowing the detection of 14 residues of dyes and the confirmation of 13 of them. The method encompasses an oxidation step running for 11 out of the 14 analytes to transform back the leuco-base forms, already known or not, into their parent forms in order to quantify only the parent form with CCα estimated below 0.5 µg kg^{-1} for the less sensitive dyes and below 0.1 µg kg^{-1} for the most sensitive. Only ethyl violet would require further development to be better stabilized during the analysis. This simple targeted method, involving an acetonitrile extraction and a short incubation for oxidation, is very sensitive and fast. As a primary strategy, the method could be easily applied for expanding the search for fraud in aquaculture using non-authorized dyes.

In a second strategy, the results of the metabolomics study

Table 2b
Potential biomarkers in liver between control trout and VPBO or MG treated trout.

MG liver						
Feature	Trend regulation	*m/z*	Rt (min)	Formula	Mass error (ppm)	Putative metabolite
M317T683	up	317.2007	11.38	$C_{22}H_{24}N_2$	1.56	*N*-Desmethyl LMG
M303T592	up	303.1850	9.87	$C_{21}H_{22}N_2$	1.81	*N*-Di-desmethyl LMG
M389T523	up	389.2328	8.71	$C_{25}H_{28}N_2O_2$	−27	Glycine conjugate-LMG
M331T762	up	331.2162	12.70	$C_{23}H_{26}N_2$	1.98	Leuco MG
M345T585	up	345.1955	9.75	$C_{23}H_{25}N_2O$	1.62	*N*-Hydroxyl MG
M730T410	up	730.3820	6.83	/	/	/
M669T405	up	669.3597	6.75	/	/	/
M720T539	up	720.4309	8.97	/	/	/
VPBO liver						
Feature	Trend regulation	*m/z*	Rt (min)	Formula	Mass error (ppm)	Putative metabolite
M480T795	up	480.3364	13.25	$C_{33}H_{42}N_3$	1.79	Leuco VPBO
M394T529	up	394.2269	8.81	$C_{27}H_{28}N_3$	1.84	*N*-Tri-desethyl VPBO
M452T715	up	452.3051	11.92	$C_{31}H_{37}N_3$	1.84	*N*-Desethyl LVPBO
M366T465	up	366.1957	7.74	$C_{25}H_{24}N_3$	1.68	*N*-Tetra-desethyl VPBO
M449T545	up	449.3477	9.07	$C_{26}H_{43}NO_5$	0.75	Glycodeoxycholic acid
M424T701	up	424.2739	11.67	$C_{29}H_{33}N_3$	1.74	*N*-Di-desethyl Leuco VPBO
Common						
Feature	Trend regulation	*m/z*	Rt (min)	Formula	Mass error (ppm)	Putative metabolite
M677T533	up	677.3625	8.87	/	/	/
M554T625	down	554.3235	10.41	/	/	/
M647T434	up	647.3778	7.23	/	/	/

presented in this article could also be considered to enlarge the targeted method. The comparison of fingerprints attached to treatments of fish by two dyes, malachite green (MG) and victoria pure blue bo (VPBO) led to interesting potential biomarkers. These two dyes are relatively close in their chemical structure both derived from the triarylmethanes. Then, it was assumed that a similarity in the fingerprints may arise between the two treatments. Results from PLS-DA statistical analyses permitted to constitute a final list of potential markers, especially in muscle and in liver, starting from numerous many spectrometric signals acquired by an LTQ-Orbitrap instrument. Direct exogenous metabolites of each triarylmethane like LMG or *N*-desethyl LVPBO could serve as biomarkers but for each treatment specifically. LMG is already considered as a relevant marker in the control methods for detecting illegal treatment. From our study, the metabolites of VPBO, as reduced form of leuco-bases and their desethyls, have been determined for the first time in muscle and liver of farmed fish. In addition, two endogenous biomarkers were formally identified as specific bile acids. But none of them carries a common effect on the metabolism of trout. Taurodeoxycholic acid appears to be down regulated by MG whereas glychodeoxycholate is up-regulated by VPBO. No common endogenous biomarker from dye treatment was identified by this study, but an effect of dyes on bile acid metabolism is suggested. For this reason, the second strategy proposed would be to modify and extend the targeted method with including these biomarkers. Then the targeted method would be adapted for screening the 13 targeted dyes but some biomarkers (endogenous and less selective as bile acids, or exogenous and more selective to VPBO treatment). The method could then serve for example in such project we would need to collect more data for evaluation of risk exposure. In this latter last case, the advanced targeted method could be proposed, not as a control method, but as a surveillance method. Before such implementation, further demonstration on routine positive samples would be necessary to confirm the VPBO metabolism and the bile acid biochemical modifications in farmed fish.

Declaration of Competing Interest

Estelle Dubreil and all the authors declare that they have no conflict of interest.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.05.056.

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Corrigendum

Corrigendum to "Dye residues in aquaculture products: Targeted and metabolomics mass spectrometric approaches to track their abuse [Food Chem. 294 (2019) 355–367]

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The authors regret that the Fig. 3 of the original paper (FC 294 (2019) 355–367) for MG was incorrect and corresponded to the same figure as for VPBO. The correct Fig. 3 is:

The authors would like to apologise for any inconvenience caused.

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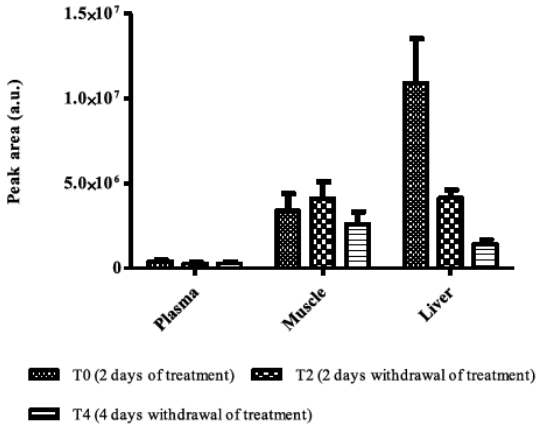
VPBO:



MG:

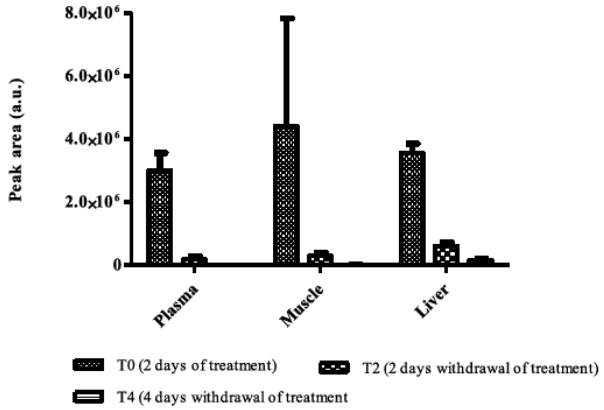


Fig. 3. Surface time profile of MG and VPBO in the different matrices during treatment and after end of treatment.